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Restoring microRNA-499-5p Protects Sepsis-Induced Lung Injury Mice Via Targeting Sox6

Wenjie Zhang[1], Jing Li[2], Hui Yao[1] and Tianmin Li[1*]

Abstract

Background: MicroRNAs (miRs) are known to participate in sepsis; hence, we aim to discuss the protective effect of miR-499-5p targeting sex-determining region Y-related high-mobility-group box 6 (Sox6) on sepsis-induced lung injury in mice.

Methods: The sepsis-induced lung injury model was established by cecal ligation and puncture. The wet/dry weight (W/D) ratio, miR-499-5p, Sox6, Caspase-3 and Caspase-9 expression in lung tissues of mice were tested. Lung injury score, collagen fibers and the degree of pulmonary fibrosis in lung tissues were determined. Further, the cell apoptosis in lung tissues was measured. The inflammatory factors contents and oxidative stress indices in bronchoalveolar lavage fluid (BALF) and lung tissues were detected via loss- and gain-of-function assays. The targeting relation between miR-499-5p and Sox6 was verified.

Results: W/D ratio and Sox6 were increased while miR-499-5p was decreased in lung tissues of sepsis-induced lung injury mice. Restored miR-499-5p or depleted Sox6 alleviated lung tissues pathology, reduced lung injury score, collagen fibers, the degree of pulmonary fibrosis, TUNEL positive cells, Caspase-3 and Caspase-9 protein expression and inflammatory factors contents in BALF and lung tissues as well as oxidative stress response in lung tissues of sepsis-induced lung injury mice. miR-499-5p targeted Sox6.

Conclusion: High expression of miR-499-5p can attenuate cell apoptosis in lung tissues and inhibit inflammation of sepsis-induced lung injury mice via depleting Sox6, and it is a potential candidate marker and therapeutic target for sepsis-induced lung injury.

Keywords: Sepsis-induced lung injury, MicroRNA-499-5p, Sox6, Apoptosis, Inflammatory factors, Oxidative stress

Introduction

Sepsis is a systemic inflammation that arises as a result of infection and causes multiple organ dysfunctions, containing acute respiratory distress syndrome [1]. It is characterized by profound hypotension, progressive hypoxemia, tissue damage and inflammatory changes [2]. The lung is the most critical and vulnerable organ in sepsis, and acute lung injury (ALI) is a frequent sepsis-induced inflammatory disease [3]. The severity of sepsis-induced ALI can be attributed to impaired permeability of the alveocapillary network, resulting in reduced oxygen supply [4]. Lung cell apoptosis is a critical factor in lung injury induced by sepsis [5]. The pathophysiology of sepsis-induced ALI is unknown, supportive measures and antibiotics are the only treatments available in sepsis and ALI patients, and their impact on the high mortality rates from sepsis is limited [6].

MicroRNAs (miRNAs) are small non-coding segments of endogenously expressed RNAs, consisting of

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approximately 18–24 nucleotides, which can control a wide range of cellular functions and bind to target regions of a particular gene to control their expression through inhibiting or activating mRNA translation/transcription [7]. MiR-499-5p is a recently discovered member of myosin-encoded miRNAs [8]. A study has reported that the serum levels of miR-499-5p could be utilized for indicating organ failure in patients with sepsis [9]. Another study has revealed that miR-499-5p expression is participated in sepsis-induced cardiac apoptosis [10]. The sex-determining region Y (SRY)-related high-mobility box (HMG) (SOX) family is defined by a set of genes including an HMG class DNA binding domain, whose sequence identity with SRY is greater than 50% [11]. The Sox6 transcription factor is a part of the SRY-related HMG-box family and expressed in multiple tissues during progression, where it serves a critical role in the transition from proliferating progenitor cells to functionally mature cells [12]. There is a study reporting that raised Sox6 is implicated in sepsis-induced cardiac apoptosis [10]. Studies have shown that Sox2 expression and Sox4 expression are heightened in human small-cell lung cancer [13, 14]. But the relationship between Sox6 and sepsis-induced lung injury needs further study. Thus, our study was to explore the protective effect of miR-499-5p on sepsis-induced lung injury mice by targeting Sox6.

Materials and Methods

Compliance with Ethical Standards

All animal experiments were in line with the Guide for the Care and Use of Laboratory Animal of the National Institutes of Health. The protocol was permitted by the Committee on the Ethics of Animal Experiments of Weihai Municipal Hospital, Cheeloo College of Medicine, Shandong University.

Experiment Animals

Healthy male specific pathogen-free grade BABL/c mice (aged 6–8 weeks, weighed 18–22 g) were purchased from the experimental animal center of Shandong University (Shandong, China). The environment was set at 24 ± 2 °C with 12 light–dark cycle alternately. Mice were treated with free access to aseptic food and sterile water. Experiments were started after adaptive feeding for 7 d.

Establishment of Sepsis-Induced Lung Injury Model by Cecal Ligation and Puncture (CLP) in Mice

Mice were randomly distributed into 2 groups: the sham group ($n = 8$) and the model group. Mice were fasted for 12 h before operation and then anaesthetized with 1% pentobarbital sodium (70 mg/kg) by intraperitoneal injection. The effect of anesthesia was observed; the breathing and heartbeat of mice were noticed. The head and limbs of mice were fixed in a supine position, and the abdominal skin was prepared and disinfected by iodophor cotton balls. Mice in the model group were treated with a longitudinal incision (1.0 cm) in the middle segment of the abdominal median line, the abdominal cavity was opened, and the cecum was pulled out from the abdominal cavity avoiding injuring mesenteric vessels. It was ligated with a 1–0 silk thread at 1/2 of the end of the cecum, and the cecum was punctured at the contralateral margin of the mesentery with a puncture needle No. 16. A proper amount of intestinal contents were extruded, the cecum was put back, and the incision was sutured. After opening the abdominal cavity, mice in the sham group were pulled out the cecum only, and then, the cecum was put back. Mice were subcutaneously injected with 1 mL 0.9% sodium chloride solution post-operation, separated in cages and routinely fed.

In Vivo Transfection

Before modeling, mice were assigned into 7 groups ($n = 8$): the model group, the miR-499-5p agomir group, the agomir negative control (NC) group, the small interfering RNA (si)-Sox6 group, the si-NC group, the miR-499-5p agomir + overexpression (oe)-Sox6 group and the miR-499-5p agomir + oe-NC group. An hour before modeling, mice were treated according to the grouping. Mice were anaesthetized with 1% pentobarbital sodium by intraperitoneal injection, then fixed in a 45° slope supine position, and the tongue of mice was moved to the other side of mouth to ensure their respiratory tract patency. The anterior skin of the neck of mice was disinfected and cut off to expose the trachea. Transfection complex (50 μL) was absorbed by a microsampler (dripped in accordance with the group, the same amount of phosphate-buffered saline (PBS) was dripped into the model group) and then slowly dripped into the trachea. After the transfection, the mice were rotated vertically and the transfection complex was fully distributed into the lungs [15]. During the transfection process, the respiratory and heartbeat of the mice were observed. If the respiratory and cardiac arrest occurred, the transfection was stopped immediately, and the respiratory tract was unobstructed. The transfection was continued when the breath and heartbeat of mice were stable. MiR-499-5p agomir and its NC as well as the corresponding plasmids of Sox6 were bought from Shanghai GenePharma Co., Ltd. (Shanghai, China). After transfection, mice were separated in cages and fed for 1 d, and then euthanized. Mice were anaesthetized with 1% pentobarbital sodium by intraperitoneal injection, fixed in a supine position and then euthanized by abdominal aorta venesection. The skin of mice was locally disinfected, the chest cavity was opened, the trachea and right main bronchial

were ligated, the right main bronchus was disconnected, and the right lung was removed. The lower lobe of right lung was placed in 10% neutral formalin for pathological examination, the middle lobe was quickly weighed to calculate the wet weight (W) and baked at 56℃, and the dry weight (D) was calculated after 72 h, thus W/D ratio was calculated. The right upper lobe was stored in liquid nitrogen for mRNA and protein detection. The left lung was washed three times with 0.4 mL normal saline, and about 1 mL of bronchoalveolar lavage fluid (BALF) was collected, moved to a 1.5-mL Eppendorf (EP) tube and placed on ice. BALF was centrifuged at 1500 rpm for 5 min and transferred to a new EP tube, then stored at -80℃ for detection of inflammatory factors.

In addition, the mice were divided into 3 groups ($n=10$/group): sham group, model group and miR-499-5p agomir group (50 μL miR-499-5p agomir was instilled into the trachea 1 h before surgery). The survival rate was monitored every 12 h, totally for 7 d. The survival status of the mice within 7 d was observed, and the toxicity of miR-499-5p was determined [16].

Hematoxylin–Eosin (H&E) Staining and Injury Score

Lung tissues were prepared into paraffin sections, stained with H&E and observed under an optical microscope. Lung injury was scored (alveolar congestion, hemorrhage, neutrophil infiltration or aggregation in the alveolar cavity or vessel wall, alveolar wall thickening and/or hyaline membrane formation) by the pathological scoring system for lung injury issued by the American Thoracic Society. 0 point indicated no or very mild lesions; 1 point indicated mild lesions; 2 points indicated moderate lesions; 3 points indicated severe lesions; 4 points indicated very severe lesions. The total score of the four items represented the total score of lung injury [17].

Masson Staining and Score

Lung tissues were made into paraffin sections (4 μm) and treated with Masson staining [18]. Collagen fibers, mucus, and cartilage were blue, cytoplasm, muscle, cellulose and glial were red, and cell nuclei were blue-black. In the semiquantitative Ashcroft scoring system [19], 0 point indicated normal lung; 1 point indicated mild fibrous thickening of alveolar or bronchial wall; 3 points indicated moderate thickening of alveolar or bronchiolar wall without damage to the lung structure; 5 points indicated worsening fibrosis accompanied by damage to the lung structure, forming fiber bands or masses; 7 points indicated severe lung structure damage and large areas of fibrosis, forming fiber bands or clumps; 8 points indicated all regional fibrous packing.

Terminal Deoxynucleotidyl Transerase-Mediated Deoxyuridine Triphosphate-Biotin Nick End-Labeling (TUNEL) Staining

The apoptosis of lung tissues was tested by TUNEL kit (Roche Diagnostics GmbH, Mannheim, Germany) and observed by an optical microscope. The apoptotic cells were stained brown in the nucleus. The apoptosis index indicated the percentage of TUNEL-positive cells [20].

Immunohistochemistry

The lung tissue sections were deparaffinized with xylene, incubated with 3% hydrogen peroxide and blocked with the goat serum. Then, Caspase-3 (ab13847, 1:500), Caspase-9 (ab32539, 1:250) and SOX6 (ab30455, 1:500, all from Abcam, MA, USA) were used as antibodies to immunostain sections. An optical microscope (Leica Microsystems, IL, USA) was utilized to capture images which were then analyzed with Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MA, USA) [21].

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

OMEGA kit (Solarbio science & technology Co., Ltd., Beijing, China) was adopted for extraction of total RNA in lung tissues and determination of RNA concentration. The complementary DNA (cDNA) was composed by reverse transcription in line with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA). The primers were designed by Invitrogen (Carlsbad, CA, USA), as shown in Table 1. PCR reaction was carried out in accordance with Fast-Start Universal SYBR Green Master (Rox) kit (Roche). The experiment was quantitatively analyzed by $2^{-\triangle\triangle Ct}$ method. U6 was the loading control of miR-499-5p, and GAPDH was the internal parameter of other genes [22].

Table 1 Primer sequence

Gene	Sequence (5′ → 3′)
miR-499-5p	Forward: GGGGTTAAGACTTGCAGTG
	Reverse: CAGTGCGTGTCGTGGAGT
Sox6	Forward: CCCCTCTGAACATGGTGGTGGC
	Reverse: TGAGACTGCCCCTGCCGAGT
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT
GAPDH	Forward: AGACAGCCGCATCTTCTTGT
	Reverse: CTTGCCGTGGGTAGAGTCAT

miR-499-5p, microRNA-499-5p; Sox6, sex-determining region Y-related high-mobility-group box 6; GAPDH, glyceraldehyde phosphate dehydrogenase

Western Blot Analysis

Total protein was collected using radio-immunoprecipitation assay lysis buffer to treat lung tissues. The total protein concentration was gauged by bicinchoninic acid method. The protein sample (30 μg) was processed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to the polyvinylidene fluoride membrane, blocked with 5% skim milk powder and reacted with primary antibodies Sox6 (ab30455, 1:1000, Abcam), Caspase-3 (9661, 1:1000, Cell Signaling Technology, MA, USA), Caspase-9 (9502, 1:1000, Cell Signaling Technology), tumor necrosis factor-α (TNF-α, ab6671, 1:1000, Abcam), interleukin-1β (IL-1β, 16806-1-AP, 1:500, Proteintech, USA), IL-6 (ab6672, 1:1000, Abcam) and GAPDH (sc-32233, 1:1000, Santa Cruz Biotechnology, Inc., CA, USA). Then, the membrane was incubated with the secondary antibody (1:2000) and developed by enhanced chemiluminescence [23].

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-1β, IL-6 and TNF-α in BALF were determined by ELISA kit (Boster, Hubei, China). Colorimetric determination was performed at 450 nm.

Detection of Oxidative Stress Indices

The lung tissues in liquid nitrogen were weighed and diluted in a certain proportion of saline under a low temperature condition, then homogenized and centrifuged at 4℃, 3000 r/min for 20 min at low temperature, and the supernatant was taken for determination. The activity of myeloperoxidase (MPO) was tested by MPO detection kit (NanJing JianCheng Bioengineering Institute, Nanjing, China), and malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) were tested by kits provided by JianCheng Bioengineering Institute.

Dual Luciferase Reporter Gene Assay

Sox6 may be the target gene of miR-499-5p which was predicated by a biological website (http://www.targetscan.org/vert_72/). There were four binding sites between miR-499-5p and Sox6 mRNA 3′untranslated region (UTR). The pmiR-RB-REPORT-Sox6 wild type (WT) and pmiR-RB-REPORT-Sox6 mutant type (MUT) vectors were constructed by Guangzhou RiboBio Co., Ltd. (Guangdong, China), and the corresponding luciferase reporter plasmids were generated. TC-1 cells (mice pulmonary epithelial cells, 1×10^5 cells/well, Shanghai Yiyan biological technology Co., Ltd., Shanghai, China) were seeded in a 24-well plate, and transfected with miR-499-5p agomir or agomir-NC, pmiR-RB-REPORT-Sox6 WT or pmiR-RB-REPORT-Sox6 MUT via Lipofectamine2000. Dual luciferase reporter gene assay system (Promega, WI, USA) was applied to measure luciferase activity.

Statistical Analysis

All data were analyzed by SPSS 21.0 (IBM Corp. Armonk, NY, USA) software. The measurement data were conveyed by mean ± standard deviation. Comparisons between two groups were conducted by *t* test, comparisons among multiple groups were assessed by one-way analysis of variance (ANOVA), and Tukey's post hoc test was used for pairwise comparison after ANOVA analysis. Survival curve was made by Kaplan–Meier method. *P* value < 0.05 was indicative of statistically significant difference.

Results

W/D Ratio and Sox6 are Increased While miR-499-5p is Decreased in Lung Tissues of Sepsis-Induced Lung Injury Mice

The toxicity of miR-499-5p was tested on mice to detect the survival time of septic mice. The results showed (Fig. 1a) the survival rate within 7 d of mice in the sham group was 100%, and that of septic mice was reduced. The survival rate of septic mice after miR-499-5p treatment was improved. The results of W/D ratio of lung tissues demonstrated that (Fig. 1b): versus the sham group, W/D ratio was raised in the model group ($P < 0.05$). In comparison with the agomir NC group and the si-NC group, W/D ratio was reduced in the miR-499-5p agomir group and the si-Sox6 group (both $P < 0.05$). W/D ratio was markedly heightened in the miR-499-5p agomir + oe-Sox6 group relative to the miR-499-5p agomir + oe-NC group ($P < 0.05$).

MiR-499-5p and Sox6 expression were detected and we found that (Fig. 1c–f): in contrast with the sham group, miR-499-5p reduced and Sox6 raised in the model group (both $P < 0.05$). In relation to the agomir NC group, miR-499-5p heightened and Sox6 decreased in the miR-499-5p agomir group (both $P < 0.05$). Versus the si-NC group, Sox6 depressed in the si-Sox6 group ($P < 0.05$). By comparison with the miR-499-5p agomir + oe-NC group, Sox6 dramatically elevated in the miR-499-5p agomir + oe-Sox6 group ($P < 0.05$).

The protein content of Sox6 in lung tissues of septic mice was determined by immunohistochemistry (Fig. 1g). It was observed that versus the sham group, Sox6 protein expression was increased in the Model group ($P < 0.05$); compared with the agomir NC group and si-NC group, lower Sox6 protein expression was examined in the miR-499-5p agomir group and the si-Sox6 group (both $P < 0.05$); relative to the miR-499-5p agomir + oe-NC

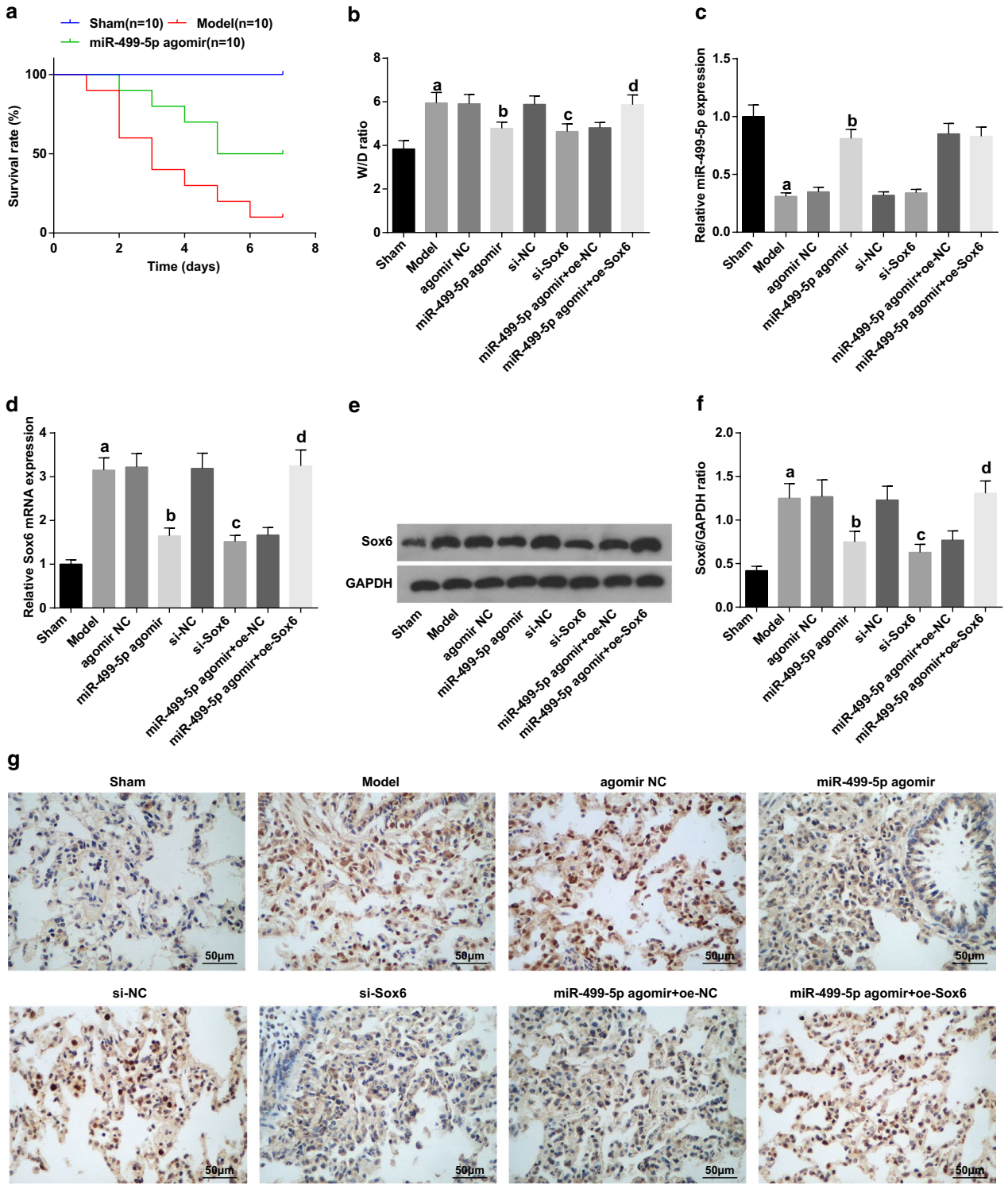


Fig. 1 W/D ratio and Sox6 are increased while miR-499-5p is decreased in lung tissues of sepsis-induced lung injury mice. **a** Survival curve of mice made by Kaplan–Meier. **b** W/D ratio of lung tissue in each group. **c** Expression of miR-499-5p in lung tissues of mice. **d** Expression of Sox6 mRNA in lung tissues of mice. **e** Protein band of Sox6 by western blot analysis. **f** Protein expression of Sox6 in lung tissues of mice. **g** Sox6 immunohistochemistry of lung tissues of mice. (a) $P < 0.05$ versus the sham group. (b) $P < 0.05$ versus the agomir NC group. (c) $P < 0.05$ versus the si-NC group. (d) $P < 0.05$ versus the miR-499-5p agomir + oe-NC group. Measurement data were depicted as mean ± standard deviation, and data were assessed by one-way ANOVA followed by Tukey's post hoc test

group, Sox6 protein expression was enhanced in miR-499-5p agomir + oe-Sox6 group ($P < 0.05$).

Restored miR-499-5p or Depleted Sox6 Alleviates Lung Tissues Pathology, Reduces Lung Injury Score, Collagen Fibers and the Degree of Pulmonary Fibrosis in Sepsis-Induced Lung Injury Mice

HE staining results presented that (Fig. 2a): in the sham group, the lung tissue structure was intact, the alveolar septum was basically manifested without edema or inflammation, and the alveolar cavity was clear. In the model, agomir NC, si-NC and miR-499-5p agomir + oe-Sox6 groups, exudation, edema and hemorrhage could be seen in most alveolar cavity of lung tissues, and massive infiltration of inflammatory cells showed in pulmonary interstitial. In the miR-499-5p agomir, si-Sox6 and miR-499-5p agomir + oe-NC groups, the lesion of lung tissues was reduced versus the model group.

Mice lung injury score and semiquantitative Ashcroft score results showed that (Fig. 2b,d): in relation to the sham group, lung injury score and the degree of pulmonary fibrosis were obviously enhanced in the model group ($P < 0.05$). Versus the agomir NC group and si-NC group, lung injury score and the degree of pulmonary fibrosis were reduced in the miR-499-5p agomir and the si-Sox6 groups (both $P < 0.05$). In contrast with the miR-499-5p agomir + oe-NC group, lung injury score and the degree of pulmonary fibrosis were markedly raised in the miR-499-5p agomir + oe-Sox6 group ($P < 0.05$).

The results of Masson staining revealed that (Fig. 2c): in the sham group, mice lung tissues contained a small amount of blue collagen fibers. In the model, agomir NC, si-NC and miR-499-5p agomir + oe-Sox6 groups, the collagen fibers were increased. In the miR-499-5p agomir group, si-Sox6 group and miR-499-5p agomir + oe-NC group, the collagen fibers were decreased versus the model group.

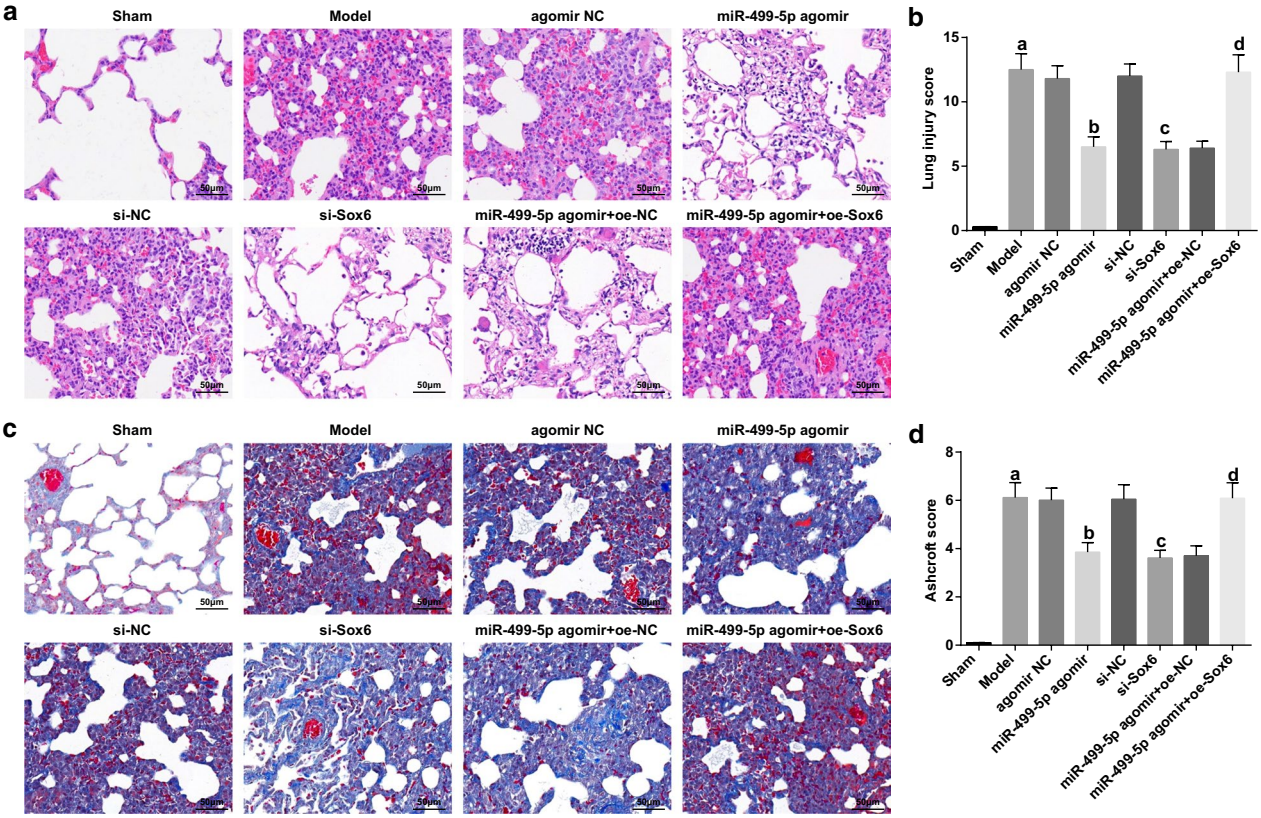


Fig. 2 Restored miR-499-5p or depleted Sox6 alleviates lung tissues pathology, reduces lung injury score, collagen fibers and the degree of pulmonary fibrosis in sepsis-induced lung injury mice. **a** Results of HE staining in lung tissues of mice. **b** The lung tissue injury score of mice in each group. **c** Masson staining of lung tissue in mice. **d** The pulmonary fibrosis scores of mice in each group. (a) $P < 0.05$ versus the sham group. (b) $P < 0.05$ versus the agomir NC group. (c) $P < 0.05$ versus the si-NC group. (d) $P < 0.05$ versus the miR-499-5p agomir + oe-NC group. Measurement data were depicted as mean ± standard deviation, and data were assessed by one-way ANOVA followed by Tukey's post hoc test

(See figure on next page.)

Fig. 3 Restored miR-499-5p or depleted Sox6 reduces TUNEL positive cells, Caspase-3 and Caspase-9 protein expression in lung tissues of sepsis-induced lung injury mice. **a** TUNEL positive cells in lung tissues of mice. **b** Number of TUNEL positive cells in lung tissues of mice. **c** Immunohistochemistry of Caspase-3 and Caspase-9 in lung tissue of mice; **d** Mean OD value of Caspase-3 and Caspase-9 in lung tissue of mice. **e** Protein bands of Caspase-3 and Caspase-9 in lung tissues of mice. **f** Caspase-3 and Caspase-9 protein expression in lung tissues of mice in each group. (a) $P < 0.05$ versus the sham group. (b) $P < 0.05$ versus the agomir NC group. (c) $P < 0.05$ versus the si-NC group. (d) $P < 0.05$ versus the miR-499-5p agomir + oe-NC group. Measurement data were depicted as mean ± standard deviation, and data were assessed by one-way ANOVA followed by Tukey's post hoc test

Restored miR-499-5p or Depleted Sox6 Reduces TUNEL Positive Cells, Caspase-3 and Caspase-9 Protein Expression in Lung Tissues of Sepsis-Induced Lung Injury Mice

Western blot, TUNEL staining and immunohistochemistry results indicated that (Fig. 3a–f): normal nucleus was blue and apoptotic nucleus was brown in different shades. Versus the sham group, TUNEL positive cells, Caspase-3 and Caspase-9 protein expression were enhanced in the model group (all $P < 0.05$). Versus the agomir NC and si-NC groups, TUNEL positive cells, Caspase-3 and Caspase-9 protein expression were reduced in the miR-499-5p agomir and the si-Sox6 groups (all $P < 0.05$). Versus the miR-499-5p agomir + oe-NC group, TUNEL positive cells, Caspase-3 and Caspase-9 protein expression were heightened in the miR-499-5p agomir + oe-Sox6 group (all $P < 0.05$).

Restored miR-499-5p or Depleted Sox6 Decreases TNF-α, IL-1β and IL-6 Contents in BALF and Lung Tissues of Sepsis-Induced Lung Injury Mice

It was displayed by ELISA and Western blot assay results that (Fig. 4a–g): TNF-α, IL-1β and IL-6 contents were increased in the model group relative to the sham group (all $P < 0.05$). Versus the agomir NC and the si-NC groups, TNF-α, IL-1β and IL-6 contents were decreased in the miR-499-5p agomir and the si-Sox6 groups (all $P < 0.05$). Versus the miR-499-5p agomir + oe-NC group, TNF-α, IL-1β and IL-6 contents were raised in the miR-499-5p agomir + oe-Sox6 group (all $P < 0.05$).

Restored miR-499-5p or Depleted Sox6 Raises SOD, CAT and GSH-Px Activities as well as Reduces MDA and MPO Contents in Lung Tissues of Sepsis-Induced Lung Injury Mice

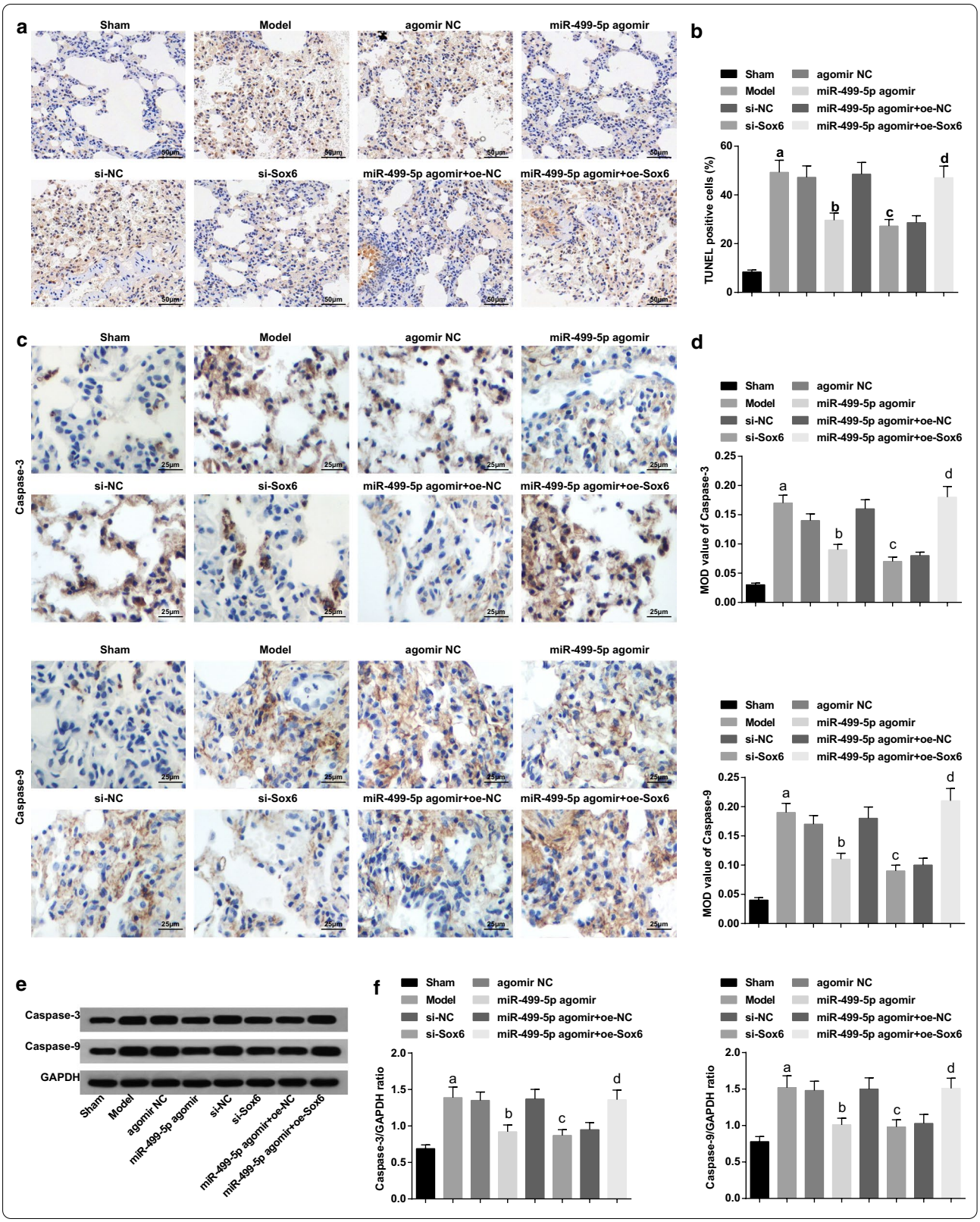
The results of oxidative stress indices detection presented that (Fig. 5a–e): versus the sham group, SOD, CAT and GSH-Px activities impaired while MDA and MPO contents raised in the model group (all $P < 0.05$). Versus the agomir NC and the si-NC groups, SOD, CAT and GSH-Px activities heightened while MDA and MPO contents reduced in the miR-499-5p agomir and the si-Sox6 groups (all $P < 0.05$). Versus the miR-499-5p agomir + oe-NC group, SOD, CAT and GSH-Px activities suppressed while MDA and MPO contents enhanced in the miR-499-5p agomir + oe-Sox6 group (all $P < 0.05$).

miR-499-5p Directly Targets to Sox6

We predicted through the bioinformatics website TargetScan that there were targeted binding sites between miR-499-5p and SOX4/6. Using RT-qPCR, it was found that compared with the sham group, SOX2/4/6 expression increased in the model group, and SOX6 expression increased mostly (all $P < 0.05$); versus the agomir NC group, the miR-499-5p agomir group demonstrated no change in SOX2 expression while showed reduced SOX4 expression, mostly decreased SOX6 (Additional file 1: Fig. S1a, b). Therefore, we finally selected SOX6 as the target gene of miR-499-5p. The biological prediction website (http://www.targetscan.org/vert_72/) revealed that miR-499-5p could target Sox6 (Fig. 6a). Dual luciferase reporter gene assay demonstrated that (Fig. 6b) versus the control group, the luciferase activity was obviously destroyed after pmiR-RB-REPORT-Sox6 WT vector and miR-499-5p agomir co-transfected into TC-1 cells ($P < 0.05$), while the luciferase activity showed no distinct difference after miR-499-5p agomir co-transfected with pmiR-RB-REPORT-Sox6 MUT vector or agomir-NC co-transfected with pmiR-RB-REPORT-Sox6 WT vector ($P > 0.05$), suggesting that miR-499-5p could directly bind to Sox6 3′UTR WT, and then inhibit the luciferase activity.

Discussion

Sepsis is a critical cause of shock and multiple organ dysfunction syndrome, among which ALI is the most frequent and significant organ complication [24]. Sepsis-induced ALI appears not only the earliest, the highest incidence, but also progresses rapidly, the fatality rate is high, and there is no effective therapy at present [25]. A previous study has purported that the up-regulation of miR-218 attenuated the secretion of inflammatory factors and prevented sepsis-induced lung injury [26]. Also, promoting the levels of miR-146a could reduce inflammation in ALI [27] and restoration of miR-125b improves the survival of mice with ALI and suppresses inflammation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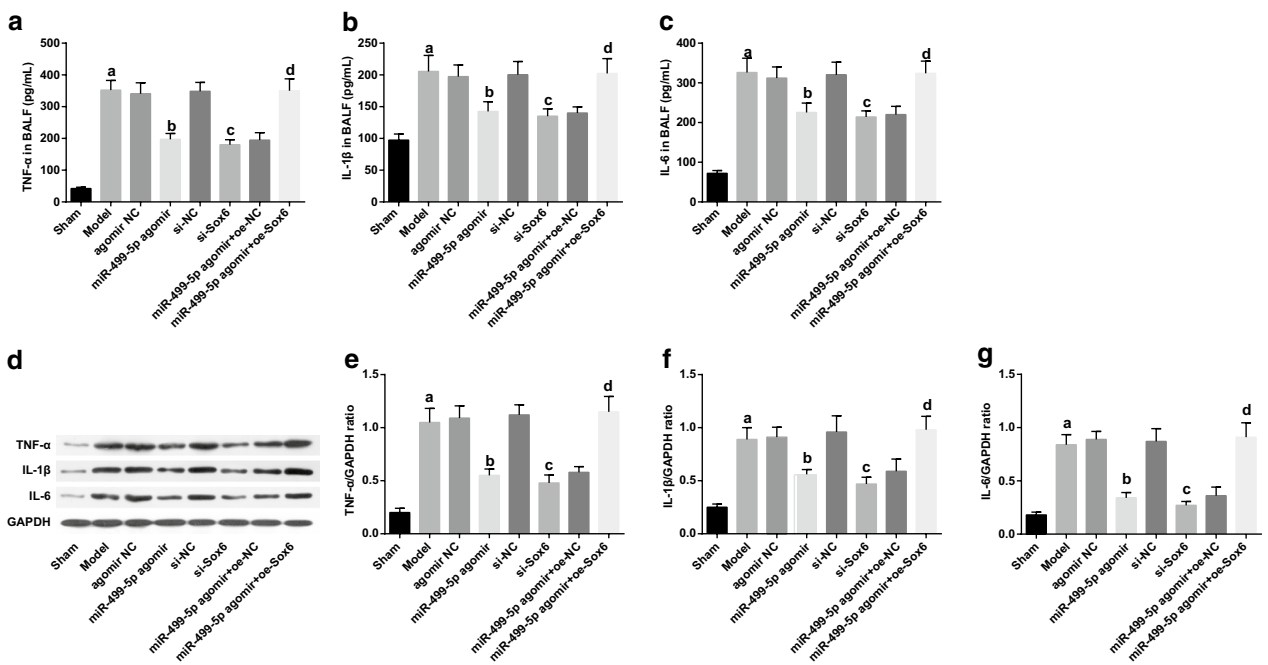

Fig. 4 Restored miR-499-5p or depleted Sox6 reduces TNF-α, IL-1β and IL-6 contents in BALF and lung tissues of sepsis-induced lung injury mice. **a** TNF-α content in BALF of mice in each group. **b** IL-1β content in BALF of mice in each group. **c** IL-6 content in BALF of mice in each group. **d** protein bands of TNF-α, IL-1β and IL-6 in lung tissues of mice in each group. **e** protein expression of TNF-α in lung tissues of mice in each group. **f** protein expression of IL-1β in lung tissues of mice in each group. **G** protein expression of IL-6 in lung tissues of mice in each group. (a) $P < 0.05$ versus the sham group. (b) $P < 0.05$ versus the agomir NC group. (c) $P < 0.05$ versus the si-NC group. (d) $P < 0.05$ versus the miR-499-5p agomir + oe-NC group. Measurement data were depicted as mean ± standard deviation, and data were assessed by one-way ANOVA followed by Tukey's post hoc test

[28]. Though studies have highlighted the role of miRNAs in lung injury, the detailed mechanism of miR-499-5p in sepsis-induced lung injury has been scarcely investigated, thus we focused on miR-499-5p to discover its therapeutic potential in sepsis-induced ALI. Also, a study has provided a proof that increased Sox6 was involved in sepsis-induced cardiac apoptosis [10]. Efforts should be made to deeply understand sepsis-induced lung injury. We aim to discuss the protective effect of miR-499-5p targeting Sox6 on sepsis-induced lung injury mice.

The W/D ratio of lung tissues was calculated in our study, and the expression of Sox6 and miR-499-5p in mouse lung tissues was assessed. The results showed that W/D ratio and Sox6 were increased while miR-499-5p was decreased in lung tissues of sepsis-induced lung injury mice. A recent study has proposed that miR-499-5p expression in patients with mild sepsis, severe sepsis and septic shock was dramatically decreased relative that in normal controls [9]. Another study has presented that miR-499-5p expression was markedly declined in non-small cell lung cancer (NSCLC) tissues and linked to poor clinical outcomes [29]. It is reported that the Sox6 expression was raised in lung cancer tissues in comparison with normal tissues [30]. Similarly, a previous study has revealed that the expression of Sox6 was remarkably elevated in the kidney tissues of mice with diabetic kidney disease [31]. Our study also presented that miR-499-5p directly targeted to Sox6. An important finding was that Sox6 is a target gene of miR-499 [10]. Another study provided data of that Sox6 was a target gene of miR-499-5p, and restoration of miR-499-5p suppressed the expression of Sox6 [32]. However, the target relation of miR-499-5p and Sox6 in lung tissues of sepsis-induced mice has not been uncovered yet.

In addition, it was revealed that restored miR-499-5p or depleted Sox6 alleviated lung tissues pathology, reduced lung injury score, collagen fibers and the degree of pulmonary fibrosis, and reduced TUNEL positive cells, Caspase-3 and Caspase-9 protein expression in lung tissues of sepsis-induced lung injury mice. It has been previously suggested that up-regulating miR-499-5p suppresses cell proliferation and promotes apoptosis in vivo and in vitro of NSCLC [29]. Another study has verified that restoring miR-499-5p and depleting Sox6 attenuated hypoxia/re-oxygenation-induced cell apoptosis and reduced Caspase-3 expression [32]. It was presented that down-regulating Sox6 alleviates the pathological injury and represses neuronal apoptosis in hippocampal tissues

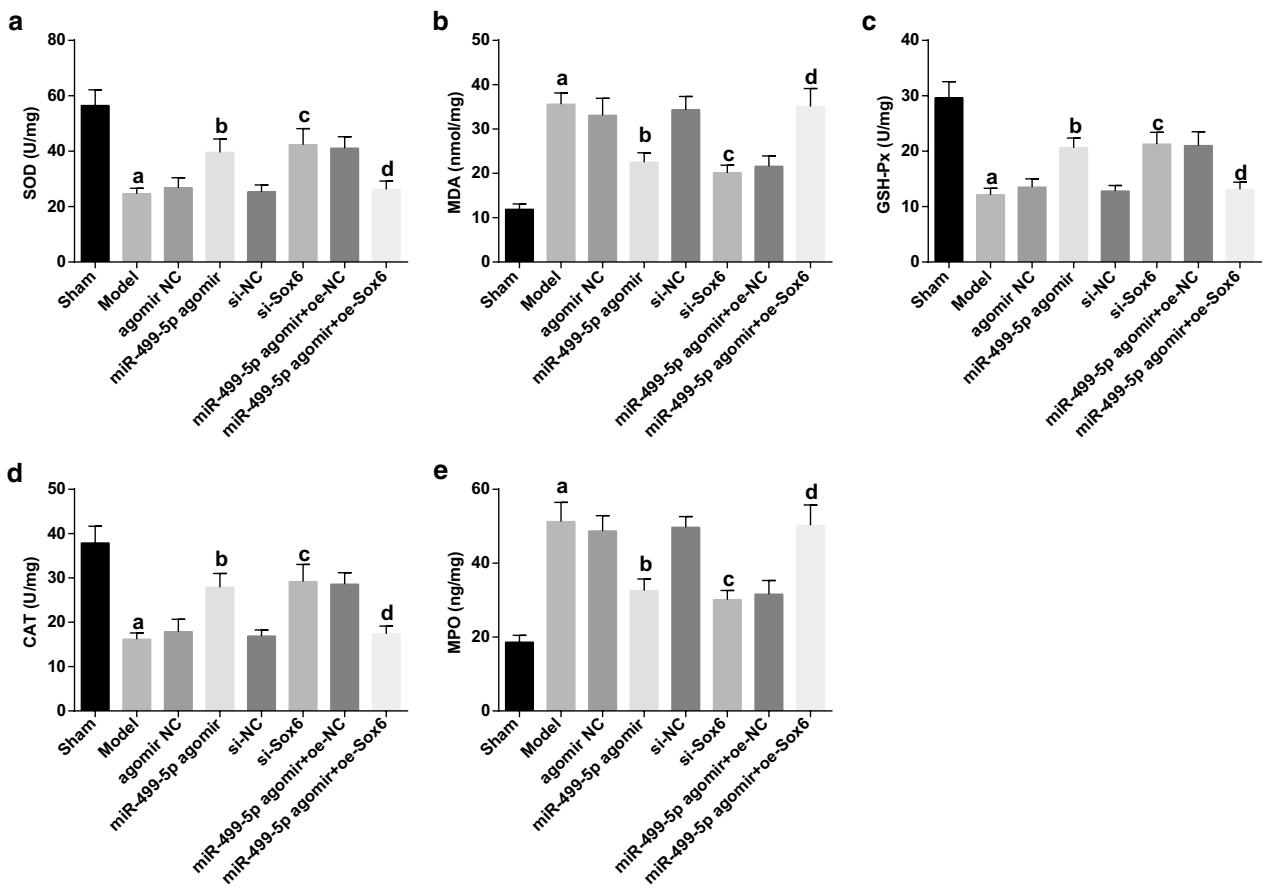

Fig. 5 Restored miR-499-5p or depleted Sox6 raises SOD, CAT and GSH-Px activities as well as reduces MDA and MPO contents in lung tissues of sepsis-induced lung injury mice. **a** SOD activity in lung tissues of mice. **b** MDA content in lung tissue of mice. **c** GSH-Px activity in lung tissues of mice. **d** CAT activity in lung tissues of each group of mice. **e** MPO content in lung tissues of mice. (a) $P < 0.05$ versus the sham group. (b) $P < 0.05$ versus the agomir NC group. (c) $P < 0.05$ versus the si-NC group. (d) $P < 0.05$ versus the miR-499-5p agomir + oe-NC group. Measurement data were depicted as mean ± standard deviation, and data were assessed by one-way ANOVA followed by Tukey's post hoc test

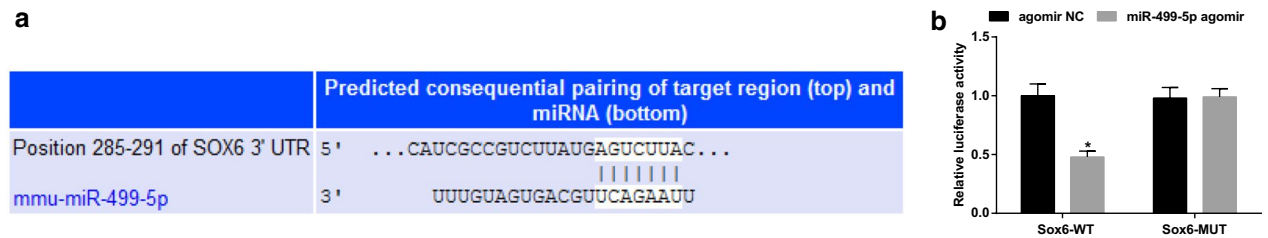

Fig. 6 miR-499-5p directly targets to Sox6. **a** The target relationship between miR-499-5p and Sox6 predicated by biological website. **b** The verification results of dual luciferase reporter gene assay. *$P < 0.05$ versus the mimic NC group. Measurement data were depicted as mean ± standard deviation, and comparisons between two groups were conducted by *t* test

of Alzheimer's disease [33]. Moreover, a study revealed that depletion of Sox6 inhibited high glucose-induced cell apoptosis and renal interstitial fibrosis in mouse renal mesangial cells [31]. Another study demonstrated that low expression of Sox6 declined the activity of Caspase-3 and the percentage of apoptotic cells in mouse P19CL6 cells [34]. In addition, we verified that restored miR-499-5p or depleted Sox6 declined TNF-α, IL-1β and

IL-6 contents in BALF and lung tissues of sepsis-induced lung injury mice. Xia et al. illuminated that TNF-α and IL-6 levels were dramatically raised in sepsis-induced lung injury mice [35]. A study has demonstrated that low expression of Sox6 declines the inflammatory reaction in hippocampal tissues of Alzheimer's disease [33]. Another result in this study implied that restored miR-499-5p or depleted Sox6 raised SOD, CAT and GSH-Px activities as well as reduced MDA and MPO contents in lung tissues of sepsis-induced lung injury mice. It was presented that CLP-induced ALI was featured by inflammation in morphology, enhanced W/D ratio, raised protein concentration in BALF, higher level of MPO and MDA contents as well as lower level of SOD, GSH-Px and CAT activities in lungs after CLP [36]. It was suggested that the activity of SOD and CAT were decreased in sepsis-induced ALI in mice [37]. Furthermore, a study reported that the overexpressed miR-499-5p and low expressed Sox6 decreased the level of MDA [32].

Conclusion

In conclusion, we found that high expression of miR-499-5p can attenuate the apoptosis of lung tissues cells and inhibit inflammation of sepsis-induced lung injury mice via depleting Sox6, which may have important therapeutic implications in the treatment of sepsis-induced lung injury. Nevertheless, clinical researches are needed to detect the efficacy for the treatment of sepsis-induced lung injury.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s11671-021-03534-x.

Additional file 1: Figure S1. Determination of Sox6 as a target of miR-499-5p in sepsis. A/B. Sox2/4/6 expression in sepsis mice. *$P < 0.05$ versus the Sham group; ^$P < 0.05$ versus the agomir NC group.

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T.L. contributed to study design; Wenjie Zhang contributed to manuscript editing; J.L. contributed to experimental studies; H.Y. contributed to data analysis. All authors read and approved the final manuscript.

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Declaration

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All animal experiments were in line with the Guide for the Care and Use of Laboratory Animal of the National Institutes of Health. The protocol was permitted by the Committee on the Ethics of Animal Experiments of Weihai Municipal Hospital, Cheeloo College of Medicine, Shandong University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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